Evaluation of role of anaerobic bacteria in gynaecological infections; such as bacterial vaginosis and infections due to long term use of contraceptive intrauterine devices

Ph.D. Thesis

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List of scientific publications related to the subject of this thesis

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2., Biofilm formation on intrauterine devices in relation to duration of use
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3., Hosszan viselt méhen belüli fogamzásgátló eszköz mikrobiológiai és Elektronmikroszkópos vizsgálata – esetismertetés
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Abbreviations

BV: Bacterial Vaginosis

IUD: Intrauterine Device

C.F.U: Colony Forming Unit

OC: oral contraceptive

STD: sexually transmitted disease

PID: pelvical inflammattory disease

HIV: human immunodeficiency virus

STI: sexually transmitted infection

Pap smear: Papanicolaou smear

SEM: scanning electron microscope

HRT: hormone replacement therapy

PIP: proline iminopeptidase

IMS: ion mobility spectometry

Contents

1. Introduction

1.1. Bacterial vaginosis

1. 1. 1. Bacterial vaginosis (BV), the most common type of vaginal infection in women of reproductive age, accounts for at least one third of all vulvovaginal infections. Earlier names for the disease include non-specific vaginitis (1), *Haemophilus* vaginitis (2), *Gardnerella* vaginitis (3), *Corynebacterium* vaginitis (4), non-specific vaginosis (5), and anaerobic vaginosis (6). The vaginal secretions of women with the disease are characterized by exponentially greater numbers of anaerobic and aerobic bacteria than are the secretions of women without the disease. BV has been related to several clinically important infectious diseases of obstetric and gynaecologic patients.

1. 1. 2. History of bacterial vaginosis

The microorganisms of the vagina have been studied since the late 1800s. *Lactobacillus* was first described as the predominant component of the normal vaginal flora in 1892 by Döederlein (7), whose name is an eponym, Döederlein's bacillus, for the organism. However, credit for characterizing and classifying the organisms belongs to Beijerinck, who gave it its current name in 1901 (8). Döederlein described three grades of vaginal cleanliness: grade I, a clean vagina, grade II, intermediate, and grade III pathological flora. Döederlein's bacilli, now observed as vaginal lactobacilli of various species, were recognized as the predominant organisms in the healthy (clean) vagina. Yeast and *T. vaginalis* were accepted as associated with vaginal signs and symptoms. A mixture of other organisms including curved, motile rods was also recognized as abnormal (pathological) flora. The syndrome associated with these organisms came to be known as „non-specific vaginitis" (NSV), to distinguish it from the specific vaginitis that were due to yeast and *Trichomonas*

(1). Gardner and Dukes characterized the syndroma of NSV clinically. The name of the syndrome was subsequently changed to BV to reflect the association with bacteria, as opposed to parasites or fungi, and to focus on the increased discharge without an inflammatory component. The syndrome has also been reported to as anaerobic vaginosis (6).

1. 1. 3. Microbiology of bacterial vaginosis

A dynamic state of population equilibrium between aerobic and anaerobic bacteria forms the normal vaginal flora. In this equilibrium, *Lactobacillus* species account for 95% of the total number of organisms. The additional 5% consist of anaerobic organisms such as *Bacteroides* species, *Prevotella bivia*, and *peptostreptococci*, as well as facultative anaerobic/aerobic organisms such as *Gardnerella vaginalis, Staphylococcus epidermidis*, and *Streptococcus* species (9). The dominance of vaginal lactobacilli results from several factors. At the time of puberty, elevated serum and tissue estrogen levels increase the glycogen content of vaginal epithelial cells. The natural ability of vaginal lactobacilli to thrive on glycogen not only promotes their own growth but also curtails the growth of potential pathogens. The metabolism of glycogen by some species of lactobacilli produces lactic acid. This results in a normal vaginal pH of 3,8 to 4,2, which is suboptimal for the growth of *Gardnerella vaginalis* and anaerobes. Furthermore, certain species of lactobacilli produce microbial toxins such as H_2O_2, which may inhibit the growth of various microorganisms, including *Gardnerella vaginalis,* anaerobes, *Neisseria gonorrhea, Chlamydia trachomatis,* and *Trichomonas vaginalis* (9). A reduced concentration of normally abundant *Lactobacillus* species and a concomitant increased concentration of Gram-negative bacteria, such as *Gardnerella vaginalis, Mobiluncus* and *Bacteroides* species, *Prevotella* and *Mycoplasma* species characterize the pathologic flora in BV. A cascade of events begins with a reduction in the number of *Lactobacillus* species, resulting in the rise of vaginal pH. This increase in pH

favors the growth of *Gardnerella vaginalis*, which decreases the oxygen concentration. This relatively anaerobic environment favors the growth of anaerobic bacteria. So microbiologically, BV is charecterized by a shift in a normal vaginal flora of *Lactobacillus spp.* to a mixed anaerobic flora. The bacterial load also increases from 10^7 to 10^{10} c.f.u./ml of vaginal fluid (10). Possible causes in the change of the flora include administration of antibiotics, use of vaginal medications, systemic hormones, contraception preparations, douches, sexual intercourse, and sexually transmitted diseases (STDs). The pathogenetic mechanism for BV is unknown, but it may vary from the use of topically applied substances with antimicrobial effect to alocalized immunologic defect or it may result from infection with an organism that competes or interferes with the lactobacilli.

1. 1. 4. Epidemiology of bacterial vaginosis

As the most common cause of vaginitis, BV affects approximately 10-25% of patients in general gynaecologic and obstetrics clinics and in up to 64% of patients visiting STD clinics (11). Similar to trichomoniasis, 50% of women with BV are asymptomatic (11). Reproducible scientific evidence supporting the sexual transmission of BV is scant despite the higher disease prevalence among patients in STD clinics relative to that of the general population. Improvements in cure rate or reduction in recurrence rates after treatment of the partner can often be observed in sexually transmitted diseases. However, multiple studies have failed to demonstrate this effect in patients with BV. In addition, identical prevalence rates of BV have been found in sexually active adolescents and virginal adolescents (12). Some studies have shown a higher relative risk of acquiring BV in women with more sexual partners. BV is very common among lesbian women. Oral sex appears to be a risk factor for heterosexual and lesbian acquisition (13). Obstetrical sequelae of BV include: preterm birth, low birth weight, chorioamnionitis, and amnionic fluid infection (14, 15,16). Non-obsterical

infections incude postpartum endometritis, pelvic inflammatory disease, cuff cellulitis, neonatal scalp abscess, non-puerperal breast abscess and cervicitis (17). BV increases the acquiring of HIV infection (18).

1. 1. 5. Clinical manifestations, differential diagnosis

Malodorous, fishy vaginal discharge is the most common symptom. The odor often exacerbated during menses and following intercourse. The alkaline nature of blood and semen favors the release of volatile amine byproducts of the anaerobic bacteria. The vaginal discharge is typically thin, dark or dull gray, homogenous and often frothy. Vulvar pruritus and irritation, common to yeast vaginitis, are rare. From the aspect of differential diagnosis, we should mention acute vaginitis and chronic vaginitis. In acute vaginitis the most common symptom associated with the infection of the vagina is discharge. Increased vaginal discharge is associated with an identifiable microbiological cause in 80 to 90% of cases. Hormonal or chemical causes account for most of the remaining cases. Three infective agents are responsible for most vaginal infections. BV accounts for 50% of infections; fungi (candidiasis) and protozoa (*T. vaginalis*) each account for about 25% (19).

In addition, considering the three most common causes above, one (chronic vaginitis) must also evaluate alternative explanations for persistent symptoms. *Chlamydia* infection may present as vulvovaginitis. Overgrowth of *Lactobacilli* is a common cause of cyclic vulvovaginitis. Although the pathophysiology of this disorder has not been fully clarified, it is postulated that the overgrowth of the lactobacilli in the vagina may result in increased vaginal acidity, with subsequent cytolysis, vaginal discharge, and vulvovaginal irritation. *Desquamative inflammatory vaginitis* is characterized by purulent discharge, exfoliation of epithelial cells, and vulvovaginal burning and erythema. This condition is generally due to a relative absence of lactobacilli and overgrowth of Gram-positive cocci (19).

1. 1. 6. Diagnosis

1. 1. 6. 1. Clinical diagnosis

The criteria developed by Amsel *et al.* in 1983 (20) are the current standard method for diagnosing BV. Clinically the diagnosis is made by identifying three of the following four findings:

1: thin, dark (dull) gray, homogenous, malodorous discharge that adheres to the vaginal wall,

2: elevated vaginal pH of greater than 4,5

3: positive whiff/amine test and

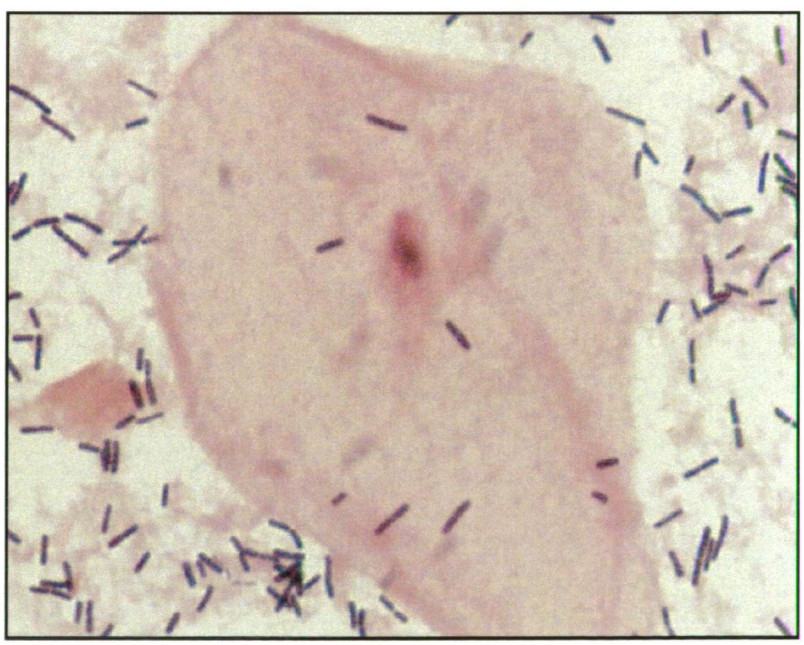
4: presence of vaginal epithelial cells (clue cells) - that are coated with small bacteria that obliterate the cell borders - on wet-mount microscopic evaluation.

Gardnerella vaginalis was subsequently identified as the predominant organism on clue cells. Epithelial cells coated with *Mobiluncus spp.* have also been observed. These criteria have a sensitivity 90% and a positive predictive value of 90% (21). Gardner and Dukes (2) who recognized the presence of clue cells on saline wet mount, first described the clinical diagnosis of BV. They also described the discharge and elevated pH. A disagreeable but not offensive odor was also noted. Pheifer et al.(22) first noted the „fishy odor" on addition of potassium hydroxide to specimens that were being examined by KOH wet mount for yeast. The specimen collected for diagnosis should be taken from the posterior fornix taken care to avoid the cervix which has an usual pH> 4.5. The „fishy odor" is due to the presence of amines in the vaginal fluid, which become volatile when the pH is increased. Trimethylamine (23) is the likely source of the odor. Putrescine and cadaverine are also present. Both: *M. curtisii* and *M. mulieris* have been shown to reduce trimethylamine oxide to trimethylamine. A microbiological source for putrescin and cadaverine among the BV-associated flora has not been found. Human cells do not manufacture these compounds.

1.1.6.2. Gram staining of vaginal fluid

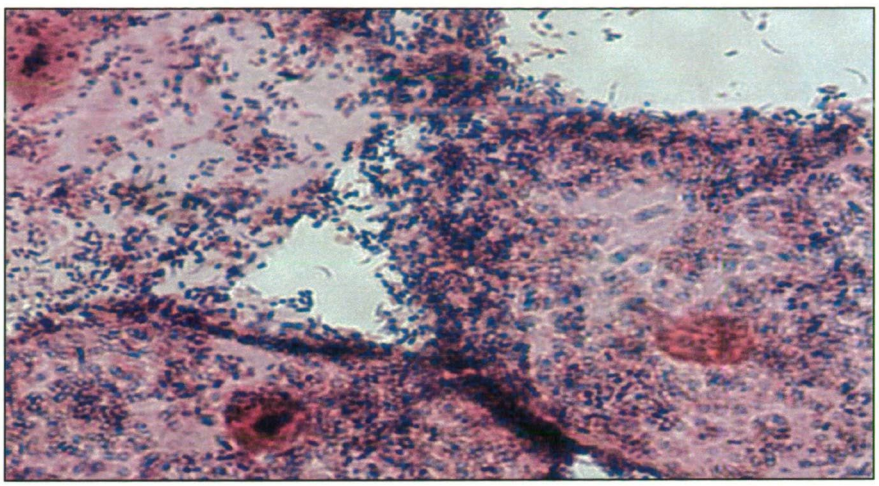
Gram stain has also been established as a reliable means for diagnosis and has the advantage of providing a permanent record. Standardized criteria developed by Spiegel *et al.* (24) yield a sensivity and specificity ranging from 62-100% and 79-100%, respectively. Nugent *et al.* (25) achieved excellent interobserver and intraobserver reproducibility of the Gram stain results by further modifying Spiegel's criteria into a scoring system. Nugent's scoring system categorizes vaginal flora as normal (Figure 1.), intermediate, or BV (Figure 2.). Women with intermedier flora have an increased risk of gonorrhoea, chlamydia, and trichomoniasis and an increased likelihood of acquiring BV when compared with women with normal flora.

Figure 1. Normal vaginal flora



Gram stain of vaginal contents (1000x) shows an epithelial cell with well-visualized borders and Gram positive rods similar to lactobacilli.

Figure 2. Bacterial vaginosis



Gram stain of vaginal discharge (1000x) from patient with bacterial vaginosis shows the borders of an epithelial cell obscured by small, Gram variable coccobacilli.

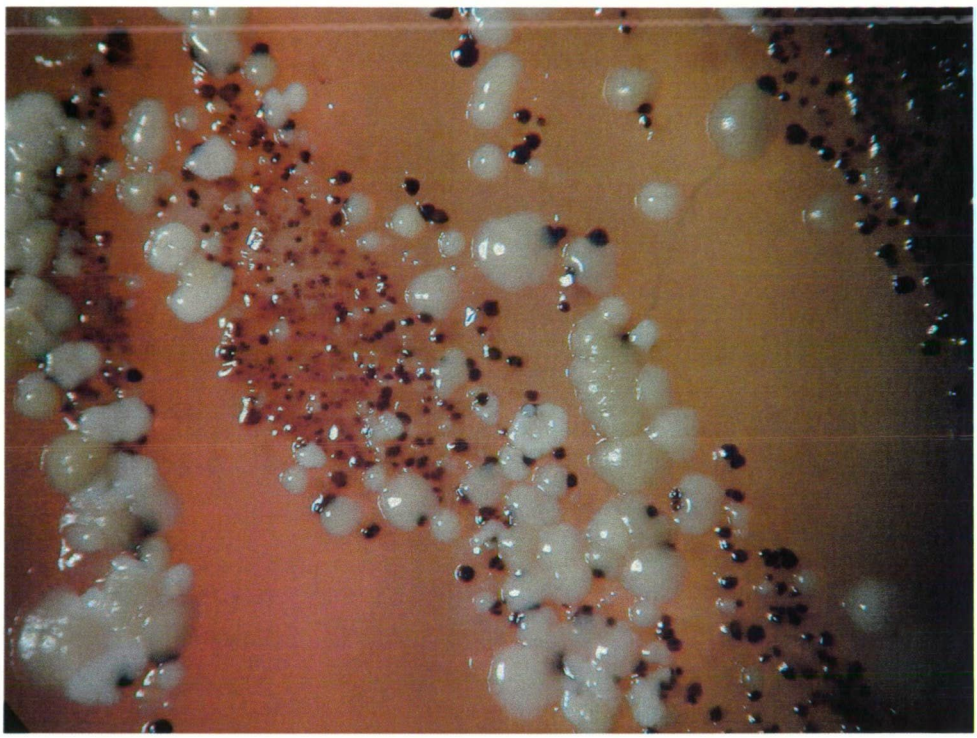
1. 1. 6. 3. Papanicolaou Smear

This technique was reported to have a sensitivity and specificity of 90 and 97%, respectively, in detecting BV when compared with clinical criteria (26). Unfortunately, these results could not be corroborated by subsequent studies. Davies et al.(27) showed that this test had a sensitivity of 55% and a specificity of 98%, making it an inadequat screening test for BV. Nonetheless, the same study also showed that Papanicolaou smears had a 96% positive predictive value. Hence, a positive Papanicolaou smear for BV obviates the need for additional confirmatory tests, but treatment can be instituted on the basis of this test alone.

1. 1. 6. 4. Culture methods

The sensitivity of a positive culture for anaerobic bacterial flora (Figure 3.) and *Gardnerella vaginalis* in patients with clinical signs of BV may be as high as 94%. However, the specificity of culture is only 50-60%, thus making it a less desirable diagnostic test. The low specificity is due to the large proportion of women with normal vaginal flora harboring *Gardnerella vaginalis*. The incidental finding of *Gardnerella vaginalis* from routine vaginal culture should never be used to diagnose BV (26).

Figure 3. Bacterial culture of typical BV vaginal flora after 5 days of anaerobic incubation in anaerobic blood agar



1.2. Intraurterine devices (IUDs)

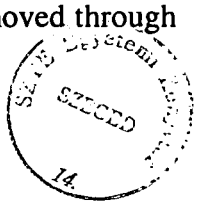
1. 2. 1. History of contraceptive intrauterine devices

Although, the history of using intrauterine devices for contraception lasts for several centuries, the pioneer of intrauterine devices (IUDs) in the modern-day considered to be Dr. Ernst Grafenberg of Germany. In the late 1920s, he developed flexible devices made of silkworm gut or coiled silver wire (28). Over the past 4 decades IUDs have played an important role in reproductive health. In the mid-1970s when IUD use peaked, nearly 10% of all women using contraceptives (approximately 2, 5 million) were using the method (29). The first four devices ever mass-marketed were made of inert plastic impregnated with barium sulfate to make them radio-opaque; the Margulies Spiral (also known as Perma-Spiral and later sold as Gynekoil) was first used in the late 1960s and was still available in the early 1970s. The Lippes Loop (1964-1986), Saf-T-Coil (1965-1984) and the Dalkon Shield came

later (30). We have to mention Professor Szontágh and the Szontágh-loop, developed by himself in the mid 1970s in Szeged. In the 1960s, researchers discovered that the effectiveness of small plastic devices could be improved by wrapping them in copper wire (30). In 1974, the first such IUD (CU-7, pronounced copper-seven) reached the US market, it's plastic frame was shaped like the number 7. Progestasert was the first hormone releasing IUD, and was sold for a quarter-century (1976-2001). The first T-shaped copper-bearing IUD was the TCu200, also known as Tantum-T, it's plastic frame was wrapped with copper wire that provided 200 mm^2 of copper surface. In 1988, a device providing 380mm^2 of copper surface (TCu380A) was marketed as ParaGard, and it is still available. The last IUD entered the market was Mirena (2001), this plastic device has a levonorgestrel-containing reservoir and releases 20ug per day of this synthetic hormone for 5 years.

1. 2. 2. Contraceptive intrauterine devices- microbiological aspects

Intrauterine devices (IUDs) are highly effective, long-term methods of contraception, however IUD use is limited to some regions due to concerns about increased risk of pelvic inflammatory disease (PID) and subsequent complications such as infertility and ectopic pregnancy. Some researchers have speculated that the presence of an IUD in the uterus may increase host susceptibility to infection, thus increasing the incidence of PID infections (31). In addition, some studies have shown that *Actinomyces* may proliferate significantly in the endocervix of women wearing an IUD (32,33). It has been shown that insertion of an IUD may contaminate the endometrial cavity with bacteria (34). Indeed, IUDs are considered to cause PID by leading vaginal and cervical bacteria into the uterus along the tail of the device. However, cultures obtained from the vagina of IUD users may or may not represent microbes present in the uterus (35). In fact, most of the microbes recovered from such cultures originated from the vagina. Therefore, bacteriological investigation of IUDs removed through



the cervix may represent contamination from the cervico-vaginal flora along with the microbial biofilm on the IUD.

The major complication associated with the use of medical implants such as IUDs, intravascular catheters or tubes is infection. Microorganisms originating from the normal flora can colonize these devices and form biofilms, consisting of layers of host cells and bacteria/fungi, embedded within a matrix material. It turned out, that these foreign materials are the most probable sites of biofilm formation (36). The main component of biofilm produced by the bacteria and/or fungi is an exopolysaccharide layer. This exopolysaccharide is the pivotal factor responsible for the behavior of biomaterial-centered infection. The biofilm bacteria are usually resistant to attack by antimicrobial agents and host phagocytes. This is one reason to explain why infections caused by these microorganisms are hard to treat without removal of the devices.

1. 2. 3. History of biofilm binding

In 1982, in clogged oil pipelines mucus-like materiel was identified as the cause of clogging, which blocked the pipelines total lumen (37, 38). Tests performed on this occasion proved that this material had been formed by *Pseudomonas aeruginosa*, which was called biofilm. Later several spots were discovered in nature, where the presence of biofilm was identified: on the surfaces of rocks in the sea, on the leaves of plants. Subsequently a number of tests were performed in the fields of biological scientists to search for the presence of biofilms. The most often encountered bacteria have so far proved to be *Enterobacter spp., Eserichia coli, Staphylococcus epidermidis, Staphylococcus aureus, Pseudomonas aeruginosa* and *Candida spp.* were published in medical biological articles from 1987 (37, 39, 40). They founded a thick layer of biofilm formation on the surface of biliary calculi removed from patients suffering from chronic cholangitis which covered the bacteria *Pseudomonas*

aeruginosa, which had been responsible for the continuus infection (41). It turned out that the so-called foreign materials found in the organisms, injured tissues, biomaterials are the most probable sites of biofilm forming (39). Biofilm systems produced by microbial adhaesion and aggregation, mediated by mucoid alginate (glycocalyx) "slime" is of great interest because of their significance in almost all biologic systems. The exopolysaccharide glycocalyx produced by bacteria is the pivotal factor responsible for the behaviour of biomaterial-centred infections. The complex exopolysaccharide acts as an ion-exchange resign for enhanced nutrition, moderates susceptibility to phagocytosis and response to antibodies, and functions in later stages of surface adhaesion, aggregation, and polymicrobial interaction. The biofilm glycocalyx produced by *Pseudomonas aeruginosa, Klebsiella spp., Staphylococcus epidermidis,* and *Candida albicans* after colonisation of the respiratory, urogenital and biliary tract is a potent factor leading to intractable infection (42, 43). The biofilm bacteria are usually resistant to attack by microbial agents and host phagocytes. To treat such an infection it is important that extracellular polysaccharide or glycocalyx production– which occur at an early stage of biofilm formation – should be inhibited. In the past years, however, long-term administration of erythromycin, clarythromycin and azitromycin in combination of anti-pseudomonas drugs as basic therapy had been used, and the prognosis of patients including those colonized by *Pseudomonas aeruginosa* has improved remarkably. Against this background, the influence of macrolides on mucoid alginate production by *Pseudomonas aeruginosa* was examined in vitro (44, 45). The major complication associated with the use of medical implants such as intravascular catheters, tubes and intra uterine devices is infection. Microorganisms can colonise these devices and form biofilms consisting of layers of cells embedded within a matrix material. Although fungal infections are less common than bacterial ones, they tend to be more serious. The pathomechanism of biofilm forming can be illustrated by enumerating the phases of forming step by step, which are the following: the

presence of inaminate, or relatively cell-free constituent, biofilm forming bacterium and biomaterial. Inoculation of a small quantity of biofilm forming bacterium, the opportunistic bacterium becoming pathogen. The resistance of the bacterium caused by biofilm is the cellular immunity of the host organism to antibiotic therapy. At the border of an injured tissue or foreign material, an intact tissue infection developes, which is caused by the necrotised tissue or the debris of the foreign material change in the cellular and possibly in the humoral immune response of the host organism caused by the foreign material (surface, debris) and the bacteria (capsule, toxin). In appropriate circumstances the primary adhesive force between the negative load bacteria and the surface of the foreign material forms the van der Waal's force. Beside this, hybrobic interaction also enhances their relation. The exopolysaccharide molecules produced in great amount further enhances the adhesions and the formation of receptor-ligand relations. The main component of biofilm is the alginate. This molecule is non-linear structured copolymer, containing B1, 4D mannuron acid and C5 epimer L-glycuron acid (46, 47). Several theories have emerged to explain the production of exopolysaccharides. According to some theories the polysaccharide molecules get into the extracellular space through exocytosis. However with this mechanism one cannot explain the production of antibodies against the polysaccharide component (48, 39). This phenomenon can be explained by a theory, when the polysaccharide molecules are taken into the extracellular space by a carrier mechanism, and occasionally not only the polysaccharide molecule peels off, but a part of the carrier protein as well. This protein would be responsible for the antigen nature of the polysaccharide molecule. The peeling of part of the biofilm may lead to embolisation, formation of secondary infections (49, 50).

2. Aims of the study

In the last decade, which saw a political and socio-economic transition after the fall of the regime, various changes in lifestyle have made their impact on the sexual habits of the Hungarian population.

The primary aims of the thesis were:

1.: to detect the correct prevalence of BV among symptomatic fertile women visiting the Department of Gynaecology of the Medical University of Szeged. Until this time no published data is known in this theme in the southern part of our country, and in Hungary also. All these women were previously tested for *Neisseria gonorrhoeae* with negative results.
2.: to detect BV related conditions among patients infected by BV.

The secondary objectives were:

1.: to examine the bacteria present on removed IUDs of different ages by using aerobic and anaerobic culture methods.

2.: in case of one patient with typical symptoms of PID beside quantitative culturing the biofilm bacteria of the removed IUD, scanning electron microscopy was also used for its visualization.

3. Materials and Methods

3. 1. Materials and methods for investigation of the prevalence of bacterial vaginosis

From 01. September 2001. until 01. April 2003. 4 611 non-pregnant, sexually active women above 16 years of age who were presented for a pelvic examination, regardless of the reason, were eligible for entry into the study. Participants completed a self-administered questionnaire. The following information was collected: age of sexual debut, sexual orientation, number of lifetime sexual partners, number of partners in the last 6 months, date of last sexual contact, contraception utilised, use of condoms with last sexual partner, history of sexually transmitted infections (STI), last menstrual period, date of last Pap smear, and Pap smear results. The patient's major complain as well as clinical findings of abnormal vaginal and/or cervical discharge, vaginal pH, and an amine odour test were recorded. The protocol and consent forms were approved by the Department of Gynaecology, Medical Faculty, University of Szeged.

3. 1. 1. Specimen collection

The clinical diagnosis of BV was made at the outpatient clinic by the gynecologist, and it was based on both the Amsel's criteria and the Gram-stain results, which were immediately available. A vaginal speculum, lubricated with water only, was inserted, and vaginal fluid was collected from the posterior fornix with two cotton swabs. One swab was used for pH determination and preparation of a slide for Gram-staining. The swab was rotated, removed, and inserted into a protective container without any liquid medium. Vaginal

swabs were rolled onto a clean glass slide, labelled with the study identification number, air dried, and were sent to the Institute of Clinical Microbiology and Gram stained. The microbial diagnosis of BV was made based on criteria described by Nugent *et al.* (25). The vaginal fluid with the second swab was sent, in aerobes/anaerobes and mycoplasma transport medium to the Institute of Clinical Microbiology for culture detection of micro-organisms. Amine odour was tested after removal of the speculum by adding 10% potassium hydroxide to the posterior lip of the speculum.

3. 1. 2. Interpretation of Gram-stained vaginal smears

In the quantity of three morphotypes detected per oil immersion field were identified and scored. For *Lactobacillus* morphotypes (i.e., Gram-positive rods), the values ranged from 0 to 4, with a score of 4 indicating that no organisms were found and a score of 0 indicating that 30 or more organisms were found in the sample. For *Gardnerella* and *Bacteroides* morphotypes (i.e., small Gram-negative rods or Gram-variable rods), values ranged from 0 to 4; a score of 0 indicated that no organisms were found, and a score of 4 indicated that 30 or more organisms were detected. For *Mobiluncus* morphotypes (i.e., Gram-negative curved rods), the values ranged from 0 to 2, with a score of 2 indicating that, 5 organisms were identified in the sample.

3.1.3. Culture methods

The vaginal discharges were cultured on Columbia agar plates supplemented with cow blood aerobically for 48 hours in parallel with Sabouraud agar, and on blood agar plates supplemented with vitamin K1 and hemin in an anaerobic chamber for 6 days. The

identification of the isolates was carried out by conventional methods and in some cases of aerobic and anaerobic bacterias by different stripps of the API-System. (BioMérieux Marcy l'Etoile, France) On CHROM agar (BD) for isolation of the yeast's *C. albicans, C. glabrata, C. tropicalis* and *C. krusei* were identified on the basis of the typical colours of their colonies on ChromAgar Candida (BD). All other fungal species were identified with ATB 32C and/or API AUX 20 (BioMérieux Marcy l'Etoile, France). In vitro antibiotic activities testing were of aerobic bacteria with agar diffusions method. The in-vitro susceptibilities of anaerobic bacteria and of various *Candida* isolates to antifungal agents were determined by the Etest method (AB Biodisk, Solna, Sweden). For isolation of *Mycoplasma hominis* (*M. hominis)* and *Ureaplasma urealyticum* (*U. urealyticum*) Mycoplasma-Duo (Sanofi Diagnostics) was used.

3. 2. Materials and methods for investigation of biofilm formation on IUDs

3. 2. 1. Patients

127 participants were recruited for our study among women who visited the Out-patient's unit of the Department of Obstetrics and Gynecology, University of Szeged, between 01. January 2001 - 31. December 2003. The mean age of the women was 41 years; ranging between 28 and 56. All participants underwent the following clinical and laboratory examination: history and physical examination (including pelvic examination) and transvaginal ultrasound. If signs and/or symptoms of genital tract infection were present patients were screened for vaginal and cervical infections, according to our microbiology laboratory's practice. The IUDs were removed under antiseptic conditions. After careful cleaning of the cervix and the vaginal wall with antiseptic solution (Braunol, B. Braun Medical AG, Emmenbrücke, Switzerland; in the case of iodic allergy Kodan Forte, Schülke&Mayr GmbH, Norderstadt, Germany), the removals were performed without

touching the vaginal wall or the opener instrument with the IUDs to prevent contamination by the vaginal flora.

A 41-year old patient with two previous live births was selected with typical symptoms of PID and with a 10-year old Copper-T IUD. After removal of the IUD, it was sent for scanning electron microscopic examination simultaneously with culturing in the microbiology laboratory.

3. 2. 2. Microbiological methods for vaginal flora assessment

From those women who had signs and/or symptoms of genital tract infection vaginal and cervical swabs were collected. The first vaginal swab was used for aerobic and anaerobic cultures; the second swab was used for detection of *Mycoplasma hominis* and *Ureaplasma urealyticum.* Vaginal swabs were directly inoculated onto: Columbia blood agar plates (Oxoid, Basingstoke, UK), which were incubated aerobically at 37 °C for 48 hours to isolate aerobic bacteria, including lactobacilli; Columbia human blood agar plates, which were incubated anaerobically at 37 °C for 5 days to isolate *Gardnerella vaginalis* and the bacterial vaginosis (BV) associated anaerobic bacteria; Thayer-Martin agar (Oxoid, Basingstoke, UK) was used to isolate *N. gonorrhoeae*, Sabouraud's agar plates (bioMérieux, S.A., Marcy l'Etoile, France), which were incubated at 37 °C for 48 hour to isolate *Candida* spp. "Mycoplasma DUO" (Sanofi Diagnostics Pasteur, Paris, France) was used for quantitative assessment of the presence of *M. hominis* and *U. urealyticum* after incubation for 48h. Isolated bacteria and fungi were identified by using classical methods and/or ATB/VITEK identification procedures (bioMérieux, S.A., Marcy l'Etoile, France). The presence of *Chlamydia trachomatis* on the cervical swab was looked for by the MicroTrak II Chlamydia antigen detection kit (Trinity, Biotech, Ireland).

3. 2. 3. Microbiological examination of the removed IUD

All cultures were commenced within 1 hour of sampling. Each IUD sample was immediately sent to the microbiology laboratory where it was placed in 10 ml reduced BHI (Brain Heart Infusion pH 7.2) broth (Oxoid, Bakingstoke, UK) and mixed on a Vortex shaker for 30s. After gentle dispersion the suspensions were diluted (10^{-1}-10^{-6}) in reduced BHI broth and 100 µl of each dilution and 100 µl of the corresponding undiluted suspension were plated immediately on selective and non-selective media. Columbia agar base (Oxoid, Basingstoke, UK) supplemented with 5% (v/v) cattle blood, haemin and vitamin K_1 was used to enumerate the total cultivatable bacterial flora, chocolate agar was used for calculation of the total aerobic bacterial flora. For the selective growing of *Enterobacteriaceae*, Endo agar (bioMérieux, S.A., Marcy l'Etoile, France) was applied. Fungi were selectively cultured on Sabouroud Dextrose agar (bioMérieux, S.A., Marcy l'Etoile, France). Black-pigmented anaerobic bacteria (*Prevotella* sp., *Porphyromonas* sp.) were isolated from KVLB (Kanamycin Vancomycin Laked Blood Agar) (Oxoid, Basingstoke, UK). CFAT (Cadmium-Fluoride- Acriflavine-Tellurit agar) (Oxoid, Basingstoke, UK) agar was used for isolation of anaerobic *Actinomyces* spp. For the isolation of anaerobic organisms and determination of the total cultivatable aerobic and anaerobic bacterial count, cultures were performed in an anaerobic chamber with an atmosphere of 90% N_2, 5% H_2, and 5% CO_2 (Bactron Sheldon Man, Oregon, USA) for 5 days at 37 °C. Isolated bacteria and fungi were identified by using classical methods and/or ATB/VITEK identification procedures (bioMérieux, S.A., Marcy l'Etoile, France).

3. 2. 4. Scanning electron microscopy

The 10-year old IUD, removed because of the symptoms of PID, was cut into 1cm stripes with sterile scissor, which was followed by the process of chemical dehydration (in 30-50-70-90 100% alcohol for 1-1 hour each, then in 30:70, 50:50, 70:30 alcohol:aceton mixture for 20-20 minutes). The samples were placed in the critical point drier in 100% aceton, and rinsed three times in liquid CO_2, then the critical point was identified, after which the samples were secured onto racks and coated in gold in a sputter coater. The examination was carried out with a Hitachi S 2400 scanning electron microscope (Tokyo, Japan) and the pictures were digitally recorded.

4. Results

4. 1. Results for bacterial vaginosis and other vaginal pathogens (Paper I.)

Out of 4 611 examined patients with symptoms of vaginal discharge 1 567 (34%) proved to be positive for bacteria, fungi or *T. vaginalis* by culture methods. Almost twenty-four percent of the positive samples harboured a typical Bv flora (mixed anaerobic bacteria) (Table 1.).

Table1. Distribution of different pathogens in 1 567 culture positive vaginal specimens

Species	No/ (%)
Mixed anaerobes (BV)	374/ (23.76)
Mycoplasma hominis	253/ (15.15)
Ureaplasma urealyticum	367/ (23.42)
Candida spp.	281/ (17.93)
Trichomonas vaginalis	447/ (28.52)
Others aerobs	312/ (19.91)

The presence of *M. hominis* and *U. urealyticum* was confirmed in 15.15% and 23.42% respectively. In 18% of the cases vaginal candidiasis was found. *T. vaginalis* was isolated in 28.52% of all culture positive samples. Nearly 20% of the cases different aerobic bacteria were found. The simultaneous presence of BV flora, *M. hominis, U. urealyticum, Candida* spp. and *T. vaginalis* and some bacteria was also found (Table 2.).

Table 2. Coexistance of other vaginal pathogens with BVs

BACTERIAL VAGINOSIS		No. 374
with	*Trichomonas vaginalis*	48
with	*Enterococcus faecalis*	36
with	*Candida spp.*	32
with	*Streptococcus agalactiae*	29
with	*Ureaplasma urealyticum*	24
with	*Mycoplasma hominis*	13

The low level of coexistence of *M. hominis* with BV in these patients may be due to their earlier empirical treatment with macrolids or tetracyclin. The distribution of anaerobic bacteria in clinically confirmed BV patients (374) can be seen in Table 3.

Table 3. Anaerobic bacteria isolated from bacterial vaginosis

Gram positive anaerobes	**No of isolates**
Peptostreptococcus spp.	225
Propionibacterium spp.	157
Bifidobacterium spp.	52
Eubacterium spp.	21
Gram negative anaerobes	
Bacteroides fragilis	158
Bacteroides spp.	46
Fusobacterium spp.	73
Prevotella spp.	198
Porhyromonas spp.	78
Mobiluncus spp.	264
Total anaerobes	1 272

On average 2.76 anaerobic bacteria could be isolated from one patient. The distribution of the *Candida* species isolated from this population is shown in Table 4.

Table 4. Distribution of the yeast isolated in 281 cases

Species	No/ (%)
Candida albicans	219/ (77.93)
Candida krusei	33/ (11.74)
Candida glabrata	19/ (6.76)
Candida tropicalis	8/ (2.84)
Candida spp.	2/ (0.71)

The most prevalent *Candida* isolate in these patients was *Candida albicans* (77.9%). However quite numerous cases were found where behind the symptoms *Candida glabrata* (6.8%), *Candida tropicalis* (2.8%) or other *Candida* species were isolated. Fluconasole resistant *C. krusei* strains were found in 33 cases.

The Gram-stained direct smears of the 4 611 samples were also evaluated (Table 5.)

Table 5. Bacterial morphotypes found by Gram stain in the case of 4 611 cases

Morphotypes	**No**
Lactobacilli	2020
No lactobacilli, no other bacteria	425
BV (typical clue cells)	347
Curved Gram-variable rods	251
Fusiform bacteria	275
Gram-variable rods	301
Gram-negative rods	172
Gram-positive cocci	301
Yeast	267

In 95% of cases where the culture was positive for *Candida* spp. the yeast were already seen in the direct smear. However fewer cases proved to be BV according to the results of the culture than was judged based on the investigation of the direct smear. The mixed aerobic-anaerobic flora cannot be always evaluated according to the direct smear morphotypes. Culture for aerobic and anaerobic bacteria of these specimens obtained from therapy resistant recurrent cases of BV or BV-like cases is advisable.

4. 2. Results of the studies on intrauterine contraceptive devices (Paper II.)

Out of the 127 patients involved in this study 10 were selected as control group with an IUD which was removed before 1 year of wearing. Due to the poor patient compliance some extremely old IUDs were also found. Among the investigated 117 patients (study group), in 63 cases (53.8 %) the supplementary reason of the removal was the age of the device. The manufacturer's recommendation for duration of an IUD in situ is 4 to 5 years, depending on the type of the device. In our material only 22 devices (18.8 %) were younger than 5 years (1-5 years, mean time in situ 2.5) (group 1). In 44 cases (37.6 %) the patients wore their device for 5 to 10 years (group 2), and in 51 cases (43.5 %) we found devices which were in situ for more than 10 years (group 3) (Table 6.). In two cases we removed 20 years old IUDs.

Table 6. Summarized culture results of 127 IUDs with different years in situ

Age of the IUD (No.)	No. of patients with BV/ no. investigated	No. of species/no. of IUD	Number of isolated species min-max	Average no. of species
Control (10)	0/10	12/10	0 - 2	1.2
< 5 years (22)	6/11	26/22	0 – 2	1.2
5– 10 years (44)	8/15	144/44	1 – 4	2.6
>10 years (51)	11/15	298/51	5 – 8	5.8

In 78 cases (66.6 %) the cause of removal of the IUD was the different degree of inflammation, including PID. In 14 cases (11.9 %) IUDs were removed because of metrorrhagia. One 41-year old patient with two previous live births visited our Out-patient's

unit with expressed lower abdominal pain. By bimanual examination bilateral adnexal tenderness, cervical motion tenderness and lower abdominal tenderness was found. She had no fever at the time of the visit, however the transvaginal ultrasound showed free pelvic fluid. After PID was diagnosed the 10-year old Copper-T IUD was removed.

In the control group 9 of 10 (90%) IUDs had a $<10^3$ CFU bacteria/sample, however in the study group 7 of 117 (5.9%) IUDs had the same low level presence of bacteria/sample. In patient group 1, 11 patients had vaginal culture results. In 6 cases the typical BV flora was found dominated by black pigmented anaerobic bacteria including *Mobilluncus* spp. No lactobacilli were present. From the 22 IUD samples 26 species were identified, the average number of species/IUD was 0.8 (Table 1). In patient group 2, 15 had symptoms or sings of vaginal discharge. Out of these 8 had typical culture result of BV. Altogether 144 species were isolated with an average number of species/IUD 2.6. In patient group 3 out of the 15 patients who had symptoms of vaginal discharge 11 had BV flora in the vaginal fluid. 298 species were isolated from the 51 removed IUDs with an average number of species/IUD 5.8.

Table 7. Shows the prevalence of the aerobic and anaerobic bacterial species and *Candida albicans* isolated from the 51 IUDs worn more than 10 years.

Table 7. Detailed culture results of 51 IUDs removed after >10 years

Species		Number of IUDs positive
Aerobe:	*E. coli*	10
	Enterobacteriaceae	15
	Lactobacillus	2
	E. faecalis	4
	S. agalactiae	6
	S. aureus	3
Anaerobe:	*Prevotella spp*	36
	Porphyromonas spp	19
	Bacteroides spp	24
	B. urealyticus	18
	Fusobacterium spp	16
	Mobiluncus spp	17
	Peptostreptococcus spp	10
	Propionibacteirum spp	8
	Bifidobacterium spp	2
	Clostridium spp	4
	Actinomyces spp	29
Others:		
	Mycoplasma hominis	39
	Ureaplasma urealyticum	26
	C. albicans	10

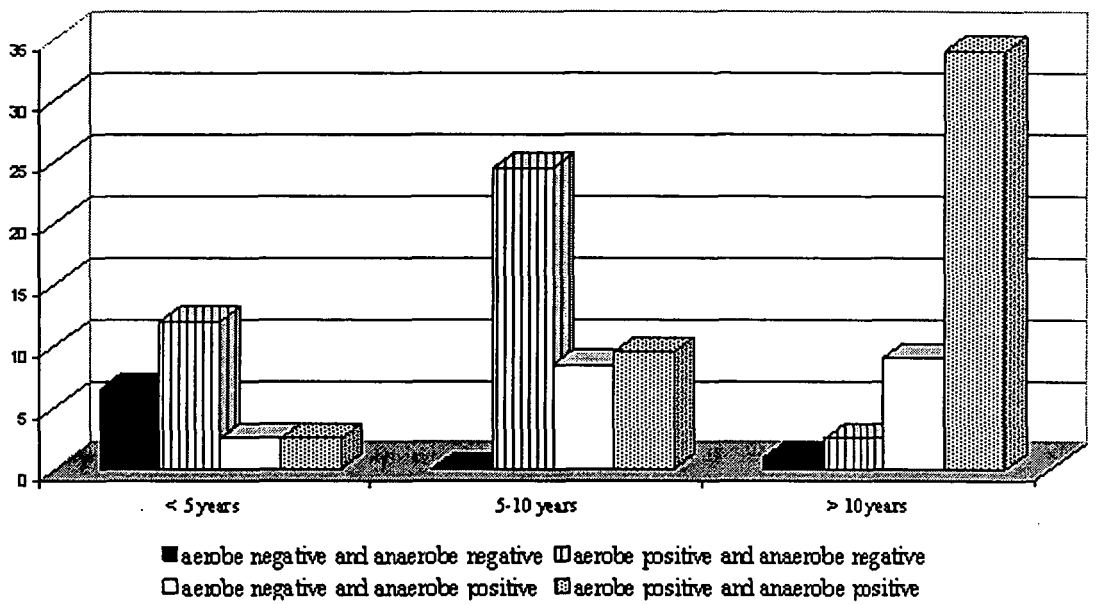
Table 8. Total CFUs of bacteria and yeasts isolated from the IUDs different ages in situ.

Total bacterial count	Number of IUDs used for years			
(CFU/sample)	<1 (control)	<5	5-10	>10
$\leq 10^3$	9	6	0	1
10^4	1	9	13	9
10^5	0	0	9	9
10^6	0	5	7	11
$\geq 10^7$	0	2	15	21

Table 8. summarises the distribution of the IUDs with different ages in situ, according to the total bacterial CFUs found during cultivation. In the case of those belonging to the control group the highest number of CFU/IUD was 10^4/sample. Low total bacterial counts were dominant among the IUDs <5 years old, however in the two other groups (IUDs removed after 5-10 years or >10 years in situ) we found significantly more IUDs with higher number of bacteria/IUD. It was remarkable that not only the CFU/IUD, but also the number and diversity of species isolated from the IUDs increased with the time in situ.

The distribution of aerobic and anaerobic bacteria on the IUDs with different ages is shown in Figure 4.

Figure 4. Percentage of the IUDs with different ages with positive cultures for aerobic and anaerobic bacteria



It is remarkable that from the biofilm on IUDs over 10 years anaerobic bacteria alone or in combination with aerobes could be isolated more frequently.

In the case of the 41-year-old patient (Paper III.), whose IUD was removed after 10 years because of symptoms of PID, a very complex anaerobic bacterial flora was isolated. The total CFU/sample was 2.5×10^8. Beside Gram-positive anaerobes (*Actinomyces viscosus, Actinomyces naeslundii, Bifidobacterium* sp, *Finegoldia magna, Anaerococcus prevotii*) Gram-negative anaerobes were dominant (*Prevotella disiens, Porphyromonas asaccharolyticus, Bacteroides ureolyticus*). One of the key pathogens in BV (*Mobiluncus* sp) was also present.

No cultures were carried out from the vaginal fluid of this patient, so it was not possible to compare the comparison of the biofilm flora with the vaginal flora. Figure 2-4. showing an immature, and a mature part of the biofilm and the surface of the IUD also.

Figure 5. Scanning electron microscopic picture from the immature part of the biofilm

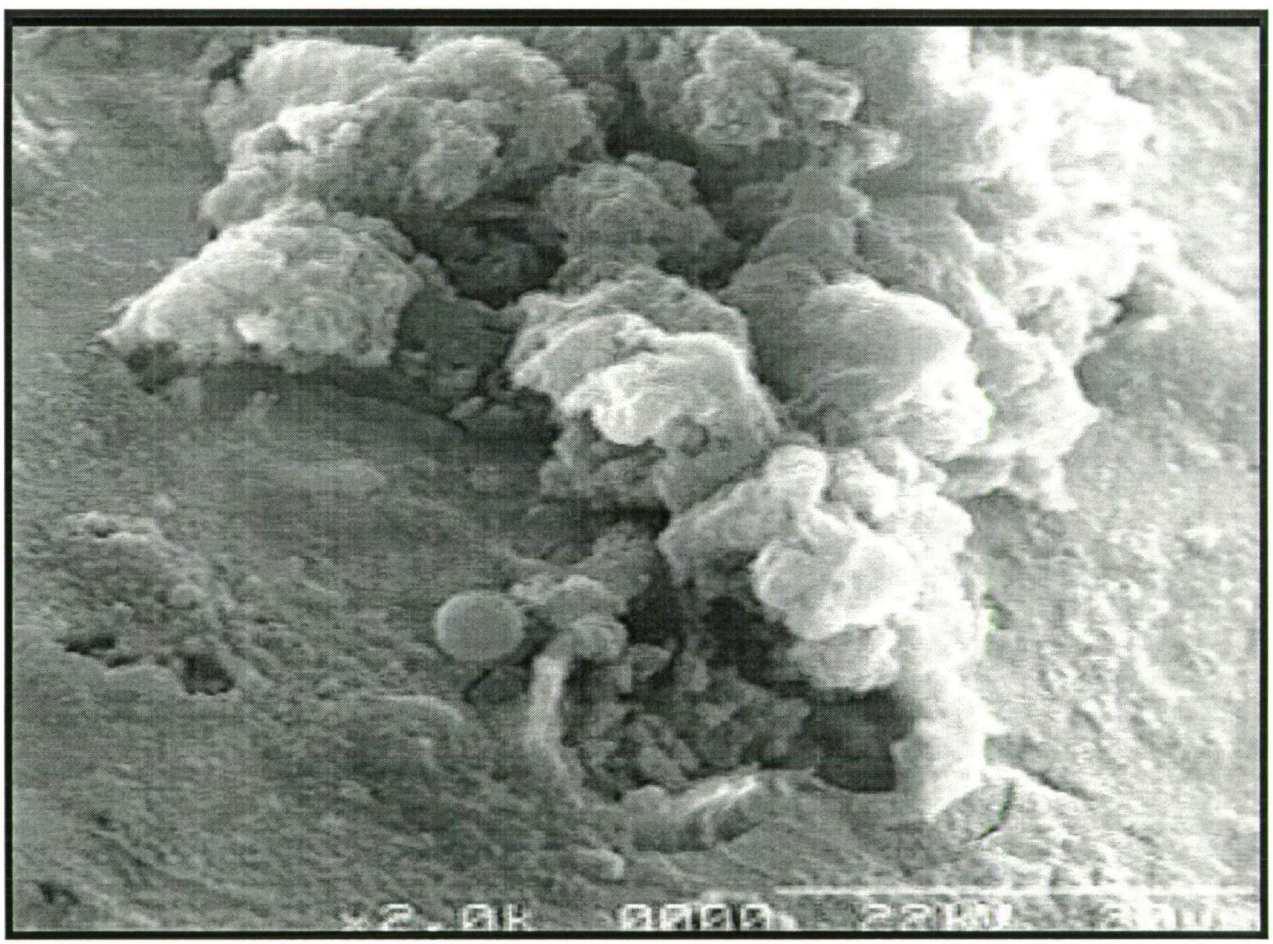


Figure 6. Scanning electron microscopic picture of mature bacterial biofilm

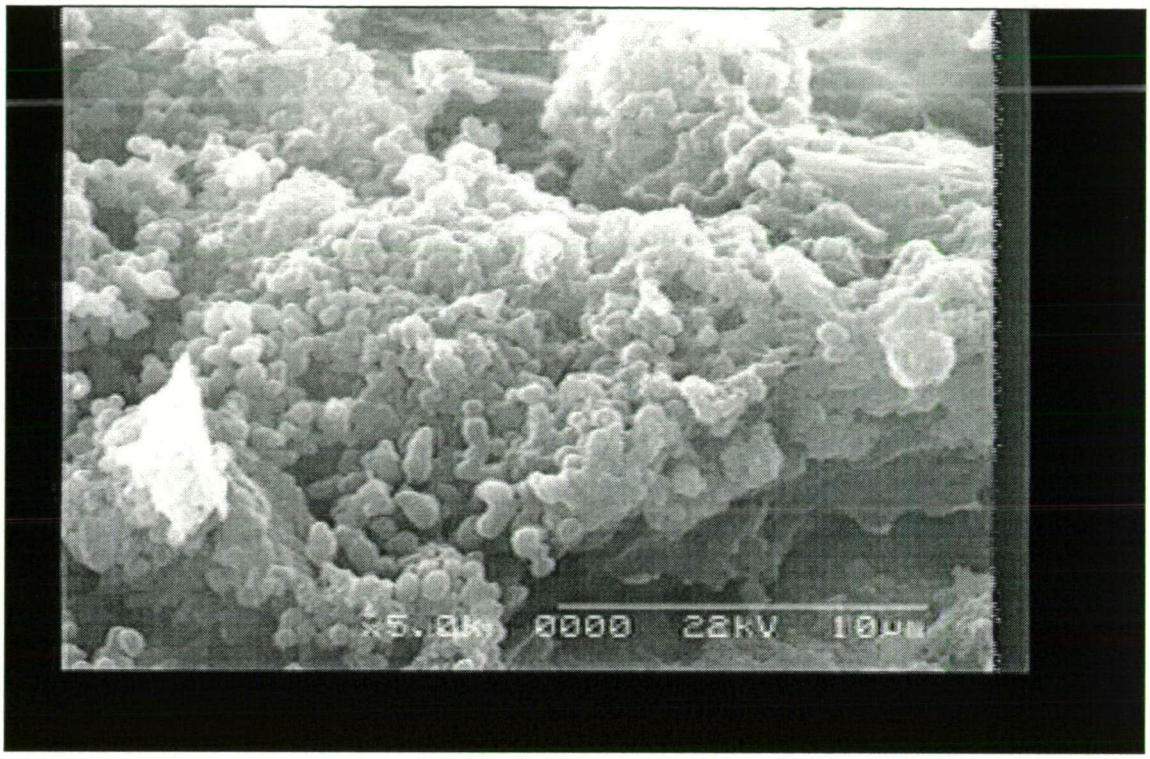
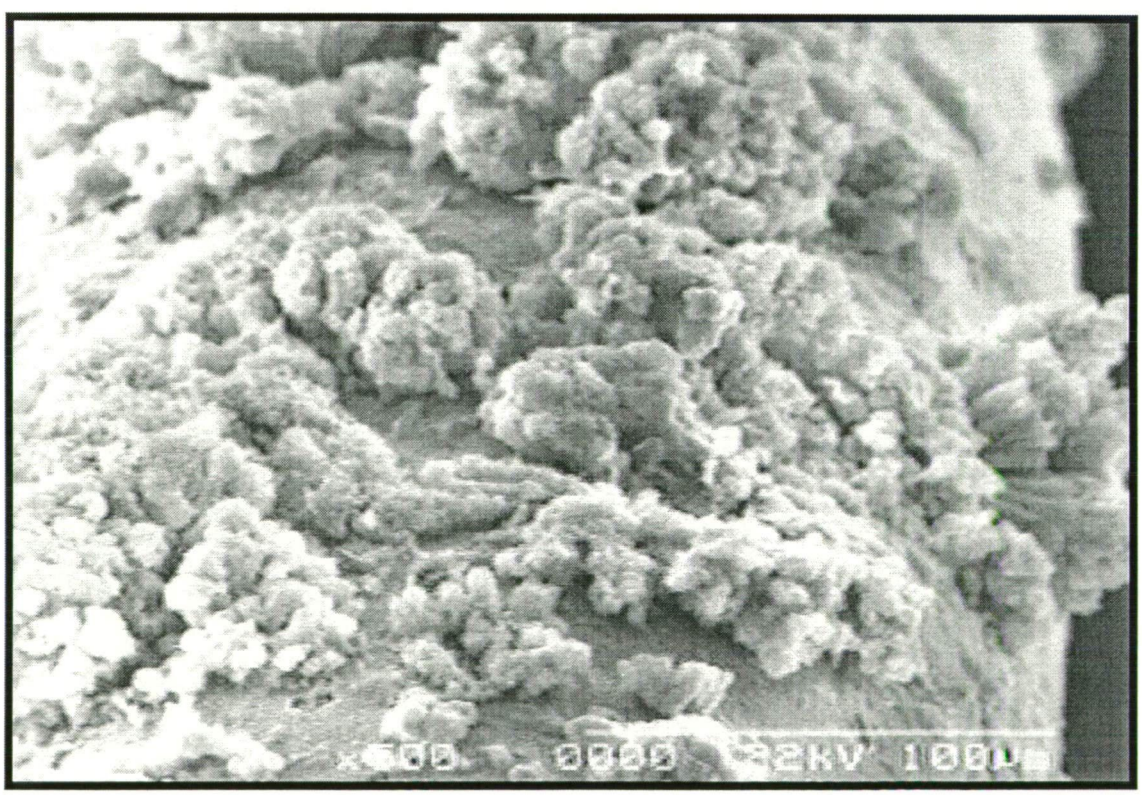


Figure 7. Around the forming biofilm the surface of the IUD can be seen



5. Discussion

5. 1. Bacterial vaginosis (Paper I.)

BV is the most frequently found pathological condition of the female genital tract. Changes in mental attitudes relating to sexual life, promiscuity, prostitution, the effects of drugs and alcohol, and the beginning of sexual activity at younger age, all involve the possibility of acquiration an infection. The disturbance in the vaginal flora frequently coexists with the inflammation of the uterine cervix. Cervical inflammation, characterized by mucopurulent discharge from the cervical os at the time of physical examination, is a condition frequently affecting young, sexually active women. Although some cases are linked to chlamydial infection or gonorrhoea, the causes of at least half are unknown. The apparent lack of a host inflammatory response to BV, as suggested by the absence of polymorphonuclear leukocytes in the vaginal fluid of women with BV, led to use of the term *vaginosis*, as opposed to *vaginitis*. However, BV may be associated with less visible inflammatory markers such as lactoferrins or various cytokines. BV has been associated with many STIs, HIV disease and cervicitis (51, 52, 53, 54, 55, 56, 57, 58). The leading hypothesis concerning these associations is that the absence of protective lactobacilli increases the biologic risk for acquiring an STI upon exposure. The mucopurulent discharge of the cervical os, oedema and easily induced endocervical bleeding can be observed. In a study by Paavonen *et al.* (59) 50% of women attending a clinic for treatment of STIs who had cervicitis, also had BV. The authors further hypothesized that cervical inflammation might predispose to altered vaginal flora. In a pilot study examining appropriate treatment for patients with BV and cervicitis, it was found that specific therapy for BV (metronidazole), along with standard antimicrobial

therapy for cervicitis, was necessary for cure of BV. It was further observed that resolution of BV was associated with the resolution of cervical inflammation (17).

Calzolari *et al.* (60) investigated a possible association between BV and the use of different contraceptive methods as compared to non-users. They didn't observe a significant difference in the BV positive group between condom or diaphragm use as compared with women without BV. Women with BV used an IUD significantly more often than women without BV, while there was a significantly negative association between BV and OC use. Similarly a negative association between condome use and the occurrence of BV was observed. They also found a significant association between previous induced abortion and BV. Their results suggest that the use of OC and condoms protect against BV, but the protection is less appreciable for the use of condoms. These findings of protective effects of OC and condom use against BV are difficult to explain. Several studies suggest that the use of OC increases the glycogen content of vaginal epithelial cells. Glycogen is a substrate for production of lactic acid by lactobacilli, which inhibit the in vitro growth of certain bacteria including BV-associated organisms. Haukkamaa et al. (61) suggests that barrier contraception with a condom prevents the anaerobic shift of vaginal flora and maintains a *lactobacilli* dominated flora. Avonts et al. (62) pointed that the tail of IUD present in the endocervix or in the vagina may favor the growth of vaginal anaerobic bacteria and *Gardnerella vaginalis*, which presumably play a role in the pathogenesis of BV.

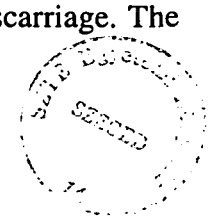
It has been suggested that the group of organisms responsible for BV may cause PID. Paavonen *et al.* (59) used a histological diagnosis of endometritis in association with clinical criteria (and confirmed by laparoscopy) to define upper genital tract infection, and found an association between this and BV based on gas-liquid chomatographic findings. Kristiansen *et al.* (1987) also showed a correlation between the isolation of *Gardnerella vaginalis* the signal bacterium of BV and endometritis. Pelvic infection is the most common but preventable cause

of infertility. The damage that occurs is usually irreversible and cannot be corrected by current techniques, except with the use of in vitro fertilization.

The incidence of PID (especially acute PID) has increased in the last few years. The most dramatic increase is in the age group between 20 and 24 years. Of these women with PID more than 20% will become fertile because of the infection and more than 3% will experience an ectopic pregnancy (63).

Schwebke *et al.* (17) found in their study, that among those women treated with metronidazole gel, those, whose BV resolved were significantly more likely to have cervicitis resolve than were those with persistent BV. The total cure rate was 70% for the BV, similar to those reported in the literature. What highlights the importance of BV is that in those cases where BV resolved by metronidazole, 100% had cervical inflammation resolve (17). The potential effects of metronidazole on cervical inflammation could be due to nonspecific antiinflammatory properties of the antibiotic or specific interactions with BV or an unrecognized pathogen. Although antiinflammatory properties of metronidazole have not been demonstrated in vitro (17), this antibiotic is widely used as adjunctive therapy along with oral antiinflammatory agents for Chron's disease. Alternatively, there may be a specific component of the largely anaerobic flora associated with BV that is pathogenic in cervical tissue and is eradicated by metronidazole.

BV does not affect conception, but is associated with an increased risk of miscarriage in the first trimester. In women undergoing in vitro fertilisation, independent of other risk factors Ralph *et al.* (56) found a significantly increased risk of miscarriage (36.1% v. 18.5%) in women with bacterial vaginosis compared with women with normal vaginal flora. This was the first study to describe a definite association between BV and miscarriage in the first trimester, and it suggests that the pathological process of BV may begin early in pregnancy. These results are genuine because they eliminated other infective causes of miscarriage. The



association between BV and miscarriage in the first trimester persisted after adjusting for other variables known to increase the risk of miscarriage-namely, smoking, older age, three or more previous miscarriages, polycystic ovary syndrome, and no previous live births. An increasing miscarriage rate was seen with increasing abnormality of the vaginal flora: 18.5% in women with normal vaginal flora, 23.3% in women with intermedier vaginal flora, and 36.1% in women with BV, as would be expected with a cause and effect relation. The pathogenesis of miscarriages with BV is not known. If endometritis was present with BV before in vitro fertilisation, this could impair implantation or early embryonic development.

A meta-analysis to evaluate BV as a risk factor for preterm delivery found that BV increased the risk of pre-term delivery >2 fold, significantly increased the risk of spontaneous abortion and maternal infection (64).

For the treatment of asymptomatic BV to prevent pre-term delivery Guaschino *et al.* (65) made a randomized trial to evaluate the effect of 2% clindamycin vaginal cream. Among women who completed the study, the rates of preterm delivery were 12.2% in the clindamycin group, and 15.7% in the no treatment group.

The idea of pH self measurement was developed by Saling at the beginning of the 1990s. The main benefit is that because of the active involvement of the pregnant women pH changes could be recognised as early as possible and as a consequence a huge part of the disorders relevant for late miscarriage or premature birth could be addressed with an immediate adaequate therapy.

In two prospective investigations, the effectiveness of the self care programme for prematurity prevention was investigated by Hoyme and Saling (66). Pregnant women have been offered to perform self measurements of their vaginal pH by means of test gloves twice a week in order to screen for any disturbances in the vaginal milieu. The women were

instructed to see their physician immediately, if abnormal pH > 4.7 or other risk factors were present, in order to get them confirmed and to start lactobacillus acidophilus therapy or in case of bacterial vaginosis to treat with clindamycin cream in the vagina. Patients who were not interested in the programme served as a control group. They found the prematurity rate 8.1% in the self measurement group, and 12.3% at the control group.

There are recent publications on new technologies for diagnosis of BV in resource-poor health care settings is often overlooked; there is a need for cheap, easy, rapid objective point-of-care diagnostic test. West *et al.* (67) tested the FemExam card, where card 1 is for pH and amines, and card 2 measures proline iminopeptidase (PIP) activity. They found the FemExam card 1 had a sensitivity of 71.4% and specificity of 72.8%, FemExam card 2 had a sensitivity of 70% and the specificity of 81%, and FemExam card 1 and 2 combined had a sensitivity of 91% and specificity of 61.5%. Chaim *et al.* (68) tried a new analytical method based on ion mobility spectrometry (IMS). It is an instrumental technique for identifying compounds and determining their concentrations, based on measurement of the velocity of ions drifting through air at atmospheric pressure under the influence of an electric field. The technique is particularly sesitive to amines taking less than 2 minutes. During the investigation the diagnostic procedure showed high accuracy and was found technically simple and rapid.

Little is known about bacterial vaginosis after the fertile period. We have only a few datas on the effect of hormone replacement therapy on the prevalence of BV. In a cohort study Cauci *et al.* (69) found that the prevalence of BV and abnormal flora in postmenopausal women are statistically lower than in premenopausal women. No significant differences in the prevalences of BV and abnormal anaerobic flora were seen in fertile and perimenopausal women. Among postmenopausal women, hormone replacement therapy (HRT) does not significantly affect the prevalence of either full BV or abnormal anaerobic flora. The absence

of lactobacilli in the absence of BV increased markedly in postmenopausal women who are not taking HRT, compared with premenopausal women – this condition must be distinguished from the partial decrease in lactobacilli associated with a limited increase in BV-associated bacteria. It was found that in women taking HRT, the colonisation of *Lactobacillus* spp. completely restored to the same level as seen in fertile women.

In our recent study, the 23.76% of culture positive cases showed typical BV flora. It is similar to the rates found in industrial countries outside the USA. Comparing these results with the results of Gram-stained smears shows that according to the other studies, culture of vaginal secretion is not as helpful as microscopic diagnosis of BV. In our results, sensitivity of culture method compared to Nugent`s method was 93% respectively.

According to Amsel's criteria, the diagnosis of BV is simple and the treatment is cheap. By restoring the normal vaginal flora we get a much better background for the cure of a possible genital infection.

5. 2. Discussion on contraceptive intrauterine devices (Paper II.-III.)

The use of IUDs is highly effective in preventing pregnancy and it is also very cost-effective. It is one of the most popular method of contraception in the world today. More than 80 million women are using IUDs for contraception world wide and the effectiveness rivals with that of tubal sterilization (31). Recent reviews suggest that the overall risk of PID with modern IUDs is lower than previously thought, at least in regions where medical advice is followed by the patients, and where low prevalence of sexually transmitted infections (STIs) exists. The risk of PID may be higher, however, in places where gonorrhoea and chlamydia are prevalent, where screening for STIs is limited and where aseptic conditions for insertion are difficult to ensure (70). It is hard to explain the reasons behind the extreme long wearing of the IUDs in a great part of our patients, but it seems to have connection with lower education and careless personal hygiene habits.

Guerreiro *et al.* (71) found significantly higher prevalence of any infection in IUD users in comparison with users of other methods as a result of higher prevalence of BV in this population. These data are in agreement with those published by Ferraz do Lago *et al.* (35) who found the prevalence of cervicovaginal infections 29.1%, and BV was frequently found (19.7%) among IUD users after 6 months of insertion. In his study he found that dysmenorrhoe and the trend of abnormal bleeding was more recurrent in patients with BV. The incidence of BV among IUD users can be even higher (47.2%) as noted by Joesoef *et al.* (72). In our study quite a few patient in all three patient groups who had symptoms or signs of genital infection had BV flora, when tested their vaginal flora. In those cases BV specific anaerobic bacteria were also found more frequently and in higher numbers on the removed IUDs as well.

There are numerous publications in the literature showing a higher risk of PID in the first weeks after IUD insertion (73, 74). The main agents responsible for PID in connection with the use of IUDs are *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, which can be present in the endocervix at the time of insertion and transferred to the upper genital tract by the device. In our case no patient was found among those who were tested with *Chlamydia trachomatis* or *Nesseria gonorrhoeae* positive results. Farley *et al.* also suggest that one reason why IUDs that have been in place for a long time are associated with more PID is that aseptic practices during insertion have significantly improved over the years, and more recently inserted devices are less likely to be infected.

Prophylactic antibiotics are not necessary to be administered routinely at the time of insertion only if the patient has positive culture (75). In a meta-analysis by Grimes *et al.* (76) the antibiotic prophylaxis for IUD insertion (either oral doxycyclin or azithromycin) significantly reduced the frequency of unscheduled return visits. The protection against PID was higher if antibiotic prophylaxis was used, but not statistically significant. In a technology assessment study, which analyzed the effectiveness of follow-up visits after IUD insertion Neuteboom *et al.* (77) compared a group of women with regular follow-up visits in the first year with women who had no regular follow-up visits. Patients in the regular follow-up visit group came more frequently for unscheduled visits. They concluded that regular follow-up after the insertion of an IUD is not effective. Patients of this study did not make big effort on following medical advice on regular follow-up visits or removal of the IUD after 4-5 years. The majority of the patients, 95 out of 117 (81%), had an IUD older than 5 years. Our data show a close correlation between the change in the number and type of the microbial flora and the proportion of patients with BV the longer the IUD was in place. This supports the recommendation for wearing the IUD for five years and not longer to ensure safety. The complexity of the biofilm flora and the dominance of the anaerobic bacteria on the IUDs older

than 5 years, were remarkable regardless whether the patient had a BV flora in their vagina or had no symptoms or signs of genital infection.

There are few recent data in the literature about the investigation of bacterial biofim formation on IUDs with different ages by quantitative culture methods. Scanning and transmission electron microscopic study of the surfaces of IUDs have already been reported by Marrie *et al.* (78). His transmission electron microscopy study showed highly organized and often densely packed micro-colonies of bacteria, given the possibility that the majority of these bacteria have been present on these surfaces for a long time. In our case a 10-year old IUD was examined for biofilm formation parallel with the culturing. An expressed biofilm formation was detected on the surface of the IUD involving both coccal and bacillary forms by scanning electron microscopy. Quantitative culture of aerobic and anaerobic bacteria showed a dominance of anaerobic bacteria in this biofilm. Bacteria living in such a biofilm are usually resistant to attack by antimicrobial agents and host phagocytes. To treat PIDs which develop in connection with the IUD wearing requires the immediate removal of the IUD and an antibiotic treatment of the inflammation active against bacteria colonizing the IUDs.

6. Conclusions

1., In the detection of the prevalence of BV among symptomatic fertile women visiting our department, we examined 4 611 patients. By culture methods 1 567 (34%) proved to be positive for pathogenic bacteria, fungi in sugnificant number, or we detected *T. vaginalis.* 374 (24%) of the positive samples harboured a typical BV flora. For the examined population the prevalence of BV was 8.11% (374). Our results seems to be the first published data for the prevalence of BV in Hungary.

2., By detecting BV related conditions we have found low level (3.47%) of coexistance of *M. hominis*, which may be due to their earlier treatment. We could isolate great number of anaerobic bacteria (2.76 bacteria/patient) either Gram positives and negatives, also. *Candida* species isolated in 281 (75.1%) patients. The most prevalent *Candida* isolate in these patients was *Candida albicans* 219 (77.9%).

3., In our material in 78 cases (66.6%) the cause of the removal was the different degree of inflammation, including PID. In the control group we have found no BV flora, and low level presence of bacteria/sample. As the period of wearing the IUD was getting longer a significant change was found in the culture results. We could detect increasing number of species/IUD, and more patients had BV flora. The species/IUD number elevated from 0.8 (<5 years) up to 5.8 (>10 years). We saw the same significant change in the total CFUs from $<10^3$ (<5 years) to $>10^7$ (>10 years).

4., The main interest of this thesis is the visualization of the bacterial biofilm on the surface of a removed IUD. After a precise preparation of the IUD stripes, the scanning electron microscoping was succesful, so we could make digitally recorded pictures of the biofilm. The immature and mature biofilm part also can be observed. The IUD's material differentiate craggy from the border of the biofilm. Such instrumental presentation with gynaecological inplant is extreme rare in international literature and it is the first time published in Hungary.

7. References

1. Krönig I. Über die Natur der Scheidenkeime, speciell über 12 das Vorkommen anaerober Streptokokken im Scheidensekret Schwangerer. Leipzig, 1892

2. Gardner HL, Dukes CD. Haemophilus vaginalis vaginitis: A newly defined specific infection previously classified „nonspecific" vaginitis. Am J Obstet Gynecol 1955;69:962-976.

3. Greenwood JR, Pickett MJ. Transfer of Haemophilus vaginalis Gardner and Dukes to a new genus Gardnerella: G vaginalis (Gardner and Dukes) comp nov. Inter J System Bacteriol 1980;30:170-078.

4. Zinneman K, Turner GC. The taxonomic position of „Haemophilus vaginalis" (Corynebacterium vaginale) J Path Bacteriol 1963;85:213-219.

5. Thomason JL, Schreckenberger PC, Spellacy WN, Riff LJ, LeBeau LJ. Clinical and microbiological characterization of patients with nonspecific vaginosis associated with motile curved anaerobic rods. J Infect Dis 1984;149:801-809

6. Spiegel CA, Amsel R, Eschenbach D, Schoenknecht F, Holmes KK. Anaerobic bacteria in nonspecific vaginitis. N Engl J Med 1980;303:601-607

7. Döederlein A. Das Scheidensekret und seine Bedeutung für Puerperalfieber. Leipzig: E. Besold;1892. Taken from Centralbl Bakteriol Parasiten 1892;11:699. German abstract

8. Beijerinck MW. Cited in Sneath PHA, Mair NS, Sharpe ME, Holt JG, eds. Bergey's Manual of Systematic Bacteriology Vol2. Baltimore, Md: Williams&Wilkins; 1986;1209-1219

9. Kimerlin DF, Andrews WW. Bacterial vaginosis: association with adverse pregnancy outcome. Semin Perinatol 1998;22(4):1-8.

10. Blackwell AL, Fox AR, Phillips I, Barlow D: Anaerobic vaginosis (non-specific vaginitis): clinical, microbiological, and therapeutic findings. Lancet 1983, ii(8364): 1379-1382.

11. ACOG. Vaginitis . ACOG Tech Bull 1996:221:1-9.

12. Mead PB. Epidemiology of bacterial vaginosis. Am J Obstet Gynecol 1993;169:446-9.

13. Berger BJ, Kolton S, Zenilman JM, Cummings MC, Feldman J, McCormack WM: Bacterial vaginosis in lesbians: a sexually transmitted disease. Clin Infect Dis 1995, 21:1402-1405.

14. Ralph SG, Rutherford AJ, Watson JD: Influence of bacterial vaginosis on contraception and misscarriage in the first trimester: cohort study. Br Med J 1999, 319:220-223.

15. Bejar R, Curbelo V, Davies C, Chick L: Pre-mature labor II Bacterial sources of phospholipase. Obstet Gynecol 1981, 57:479-482.

16. McGregor JA, French JI, Jones W, *et al.*: Bacterial vaginosis is associated with prematurity and vaginal mucinase and sialidase: results of a controlled trial of topical clindamycin cream. Am J Obstet Gynecol 1994,170:1048-1059.

17. Schwebke JR, Weiss HL. Interrelationships of Bacterial vaginosis and Cervical Inflammation. Sex Transm Dis 2001, vol29:59-64.

18. Moodley P, Conolly C, Sturm AW: Interrelatioships among human immunodeficiency virus type 1 infection, bacterial vaginosis, trichomoniasis, and the presence of yeasts J Infect Dis 2002, 185:69-73.

19. Beckman C, Ling FW, Herbert W, Laube DW, Smith RP, Barzansky BM. In: Nieginski EA, ed. Vulvitis and vaginitis. Obstetrics and Gynecology, 3rd Ed. Baltimore, MD: Williams@Wilkins, 1998:324-33.

20. Amsel R, Totten PA, Spiegel CA. Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiological associations. Am J Med 1983;74:14-22.

21. Mead PB, Eschenbach DA. Vaginitis 1998: update and guidelines. Contemp OB/GYN 1998;Jan:116-32.

22. Pheifer TA, Forsyth PS, Durfee MA, Pollock HM, Holmes KK: Nonspecific vaginitis: role of *Haemophilus vaginalis* and treatment with metronidazole. N Engl J Med 1978, 298:1429-1434.

23. Brand JM, Galask RP: Trimethylamine: the substance mainly responsible for the fishy odor often associated with vaginosis. Obstet Gynecol 1986, 63:682-685.

24. Spiegel CA, Amsel R, Holmes KR. Diagnosis of bacterial vaginosis is improved by direct gram stain of vaginal fluid. J Clin Microbiol 1983; 18:170-177.

25. Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by standardized method of gram stain interpretation. J Clin Microbiol 1991;29:297-301.

26. Hillier SL. Diagnostic microbiology of bacterial vaginosis. Am J Obstet Gynecol 1993;169:455-459.

27. Davis JD, Connor EE, Clark P,et al. Correlation between cervical cytologic results and gram stain as diagnnostic tests for bacterial vaginosis. Am J Obstet Gynecol 1997;177:532-535.

28. Thomsen RJ. An atlas of intrauterine contraception. Washington (DC): Hemisphere Publishing Corp. 1982

29. Mosher WD, Westoff CF. Trend sin contraceptive practice: United States. 1965-76. Vital Health Star 1982;23:1-47.

30. Zipper JA, Tatum HJ, Pastene L, Medel M, Rivera M. Metallic copper as an intrauterine contraceptive adjunct to the „T" device. Am J Obstet Gynecol 1969;105:1274-1278.

31. Peterson HB, Xia Z, Hughes JM, et al. The risk of pregnancy after tubal sterilization: findings from the US Collaborative Preview of Sterilization. Am J Obstet Gynecol 1996;174:1161-70.

32. Bhagavan BS, Gupta PK. Genital actinomycosis and intrauterine contraceptive devices. Cytopathologic diagnosis and clinical significance. Hum Pathol 1978;9:567-8.

33. Gupta PK. Intrauterine contraceptive devices. Vaginal cytology, pathologic changes and clinical implications. Acta Cytol 1982;26:571-613.

34. Mishell DR, Bell JH, Good RG, Moyer DL. The intrauterine device: a bacteriologic study of the endometrial cavity. Am J Obstet Gynecol 1966;96:119-26.

35. Ferraz do Lago R, Simoes JA, Bahamondes L, Camargo RPS, Perrotti M, Monteiro I. Follow-up of users of intrauterine device with and without bacterial vaginosis and other cervicovaginal infections. Contraception 2003;68:105-109.

36. Gristin GA. Biofilms and chronic bacterial infections. Clin Microbiol Newsletter 1994.16.171-176.

37. Allison DG, Sutherland IW. The role of exopolisaccharides in adhesion of fresh water bacteria. J Gen Microbiol 1987;133:1319-27.

38. Fletscher M. Adherence of marine microorganisms to smooth surfaces, in Beachy EH. (ed.) Receptors and recognition bacterial receptors. Chapman and Hall, London, 1980;347-374.

39. Gristin GA. Biofilms and chronic bacterial infections. Clin Microbiol Newsletter 1994;16:171-176.

40. Hussain M, Wilkox MH, White PJ. The slime of coagulase-negative staphylococci: biochemistry and adherence. Microbiol Rev 1993;104:191-208.

41. Hoffman BJ, Cunningham JT, Marsh WH, O'Brien JJ, Watson J. An in vitro comparison of biofilm formation on various biliary stent materials. Gastrointest Endosc 1994.40.5.581-583.

42. Anderl JN, Franklin MJ, Stewart PS. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. Antimicrob Agents Chemoter 2000.44,7.1818-24.

43. Dijk F, Westerhof M, Busscher HJ, van Luyn MJ, van Der Mei HC. In vitro formation of oropharingeal biofilms on silicone rubber treated with palladium/ tin salt mixture. J Biomed Mater Res 2000.51.3.408-402.

44. Kobayashi H. Biofilm disease: It's clinical manifestation and therapeutic possibilities of macrolides. Am J Med. 1995.99.suppl.EA.6A-29.

45. Nagino K, Kobayashi H. Influence of macrolides on mucoid alginate biosynthetic enzyme from *Pseudomonas aeruginosa*. Clin Microbiol Infect 1997.8.432-439.

46. Franklin MJ, Chitin LE, Gacesa P, Sonesson A, White DC, Ohman DE. *Pseudomonas aeruginosa* algG is a polymer level *alginate C5 mannuronan epimerase*. J Bacteriol 1994.176.1821-1830.

47. Pugashetti BK, Vadas L, Prikar HS, Feingold DS. GDP-mannose dehydrogenase and biosynthesis of alginate-like polisaccharide in a mucoid strain of *Pseudomonas aeruginosa*. J Bacteriol 1983.153.1107-1110.

48. Anwar H, Dsgupta MK, Costerton JW. Testing the susceptibility of bacterial biofilms to antibacterial agents. J Antimicrob Agents Chemoter. 1990.34.2043-2046.

49. Hoiby N, Döring G, Schiotz PO. The role of immune complexes in the patogenesis of bacterial infections. Annu Rev Microb 1986.40.29-53.

50. Hoyle BD, Jass J, Costerton JW. The biofilm glycocalyx as a resistance factor. J Antimicrob Chemoter 1990.26.1-6.

51. Barbone F, Austin H, Louv WC, Alexander WJ. A follow-up study of methods of contraception, sexual activity and rates of trichomoniasis, candidiasis and bacterial vaginosis. Am J Obstet Gynecol 1990; 163:510-514.

52. Berger BJ, Kolton S, Zenilman JM, Cummings MC, Feldman J, McCormack WM. Bacterial vaginosis in lesbians: a sexually transmitted disease. Clin Infect Dis 1995;21:1402-1405.

53. Fethers K, Marks C, Mindel A, Estcourt CS. Sexually transmitted infections and risk behaviors in women who have had sex with women. Sex Transm Infect 2000;76:345-349.

54. Schwebke CJ, Richey CM, Weiss HL. Correlation of behaviors with microbiological changes in vaginal flora. J Infect Dis 1999;180:1632-1636.

55. Hillier S, Holmes KK, et al. Sexually Transmitted Diseases 3rd edn. McGraw-Hill, New York;1999:563-586

56. Ralph SG, Rutherford AJ, Watson JD. Influence of bacterial vaginosis on coception and misscarriage in first trimester: cohort study. Br Med J 1999;319:220-223.

57. Moodley P, Connolly C, Sturm AW. Interrelationships among human immundeficiency virus type 1 infection, bacterial vaginosis, trichomoniasis and the presence of yeasts. J Infect Dis 2002;185:69-73.

58. US Department of Health and Human Services, Centers for Disease Control and Prevention, Atlanta: Guidelines for treatment of sexually transmitted diseases. MMWR 1998;47:RR-1.

59. Paavonen J, Critchlow CW, DeRouen T, et al. Etiology of cervical inflammation. Am J Obstet Gynecol 1986;154:556-564.

60. Calzolari E, Masciangelo R, Milite V, Verteramo R. Bacterial vaginosis and contraceptive methods. Int J Gyn Obstet 2000;70:341-346.

61. Haukkamaa M, Stranden P, Jousimies-Somer H, Siitonen A. Bacterial flora of the cervix in women using different methods of contraception. Am J Obstet Gynecol 1986;154:520-524.

62. Avonts D, Sercu M, Heyerick P, Vandermeeren I, Meheus A, Piot P. Incidence of uncomplicated genital infections in women using oral contraception or an intrauterine device: a prospective study. Sex Transm Dis 1990;17:23-29.

63. Dewhurst's Textbook of Obtetrics and Gynaecology for Postgraduates. Editied by Charles R. Whitfield. Blackwell Science. Fifth Edition (1995).

64. Leitich H, et al. Bacterial vaginosis as a risk factor for preterm delivery: a meta-analysis. Am J Obstet Gynecol 2003 Jul;189(1):139-147.

65. Guaschino S, Ricci E, Franchi M, et al. Treatment of asymptomatic bacterial vaginosis to prevent pre-term delivery. Eur J Obstet Gynecol 2003;110:149-152.

66. Hoyme UB, Saling E. Efficient prematurity prevention is possible by pH-self measurement and immediate therapy of threatening ascending infection. Eur J Obstet Gynecol 2004;115:148-153.

67. West B, et al. Evaluation of new rapid diagnostic kit (FemExam) for bacterial vaginosis in patients with vaginal discharge syndrome in The Gambia. Sex Transm Dis 2003 Jun;30(6):483-489.

68. Chaim W, et al. New technology for diagnosis of bacterial vaginosis. Eur J Obstet Gynecol Reprod Biol 2003 Nov 10;111(1):83-87.

69. Cauci S, Driussi S, De Santo D, et al. Prevalence of bacterial vaginosis in perimenopausal and postmenopausal women. J Clin Microbiol 2002;40(6):2147-2152.

70. Steen R, Shapiro K. Intrauterine Contraceptive Devices and Risk of Pelvic Inflammatory Disease: Standard of Care in High STI Prevalence Settings. Rep H Matters, 2004 May vol.12(23):136-143.

71. Guerreiro D, Gigante MAM, Teles LC. Sexually transmitted diseases and reproductive tract infections among contraceptive users. Int J Gyn Obstet 1998;63:S167-S173.

72. Joesoef MR, Karundeng A, et al. High rate of bacterial vaginosis among women with intrauterine devices in Manado, Indonesia. Contraception 2001;64:169-172.

73. Grimes DA. Intrauterine devices and pelvic inflammatory disease: recent developments. Contraception 1987;36:97-107.

74. Farley TMM, Rosemberg MJ, Rowe PJ, et al. Intrauterine devices and pelvic inflammatory disease: an international perpective. Lancet 1992;339:785-788.

75. Tsanadis G, Kalantaridou SN, Kaponis A, Paraskevaidis E, Zikopoulos K, Gesouli E. et al. Bacteriological cultures of removed intrauterine devices and pelvic inflammarory disease. Contraception 2002;65:339-342.

76. Grimes DA, Schultz KF. Prophylactic antibiotics for intrauterine device insertion: a metaanalysis of the randomized controlled trials. Contraception 1999;60:57-63.

77. Neuteboom K, de Kroon KD, Dersjant-Roorda M, Jansen FW. Follow-up visits after IUD-insertion: sense or nonsense?: A technology assessment study to analyze the effectiveness of follow-up visits after IUD insertion. Contraception 2003;68:101-104.

78. Marrie TJ, Costerton JW. A scanning and transmission electron microscopic study of the surfaces of intrauterine contraceptive devices.Am J Obstet Gynecol 1983;146:384-394.

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